EXHIBIT 1

APPLICATION FOR PROVISIONAL PATENT

BY

TILLMAN U. GERNGROSS

FOR

METHOD FOR MODIFYING GLYCOSYLATION OR RECOMBINANT PROTEINS EXPRESED IN YEAST

METHOD FOR MODIFYING GLYCOSYLATION OF RECOMBINANT PROTEINS EXPRESSED IN YEAST

Background of the Invention

The present invention generally relates to modification of the glycosylation of recombinant proteins expressed in yeast systems, to more closely resemble the glycosylation of proteins expressed in mammalian systems.

Many proteins are glycosylated after they are translated from the DNA encoding the protein. Different organisms express different glycosylation enzymes and have different substrates available, so that the glycosylation patterns as well as composition of the individual sugars, even of the same protein, will be different depending on the host system. Bacteria are typically unable to glycosylate proteins in any manner. Yeast add different types of sugars. Insect cells transformed with vectors such as baculovirus glycosylate proteins in still other ways. See, for example, R.K. Bretthauer, et al. *Biotechnology and Applied Biochemistry*, 1999, 30, 193-200; W. Martinet, et al. *Biotechnology Letters*, 1998, 20, 1171-1177; S. Weikert, et al. *Nature Biotechnology*, 1999, 17, 1116-1121; M. Malissard, et al. *Biochemical and Biophysical Research Communications*, 2000, 267, 169-173; Jarvis, et al. 1998 Engineering N-glycosylation pathways in the baculovirus-insect cell system, Current Opinion in Biotechnology, 9:528-533; and M. Takeuchi, *Trends in Glycoscience and Glycotechnology*, 1997, 9, S29-S35.

It is well established that humans and old world primates carry out post-translational glycosylation reactions that differ from most mammals and in particular from lower eukaryotes. However, therapeutic efficacy and pharmacokinetic properties of human glycoproteins are often highly dependent on the particular oligosacharide structure of the glycoprotein. Since the glycosylation patterns and composition affect antigenicity as well as clearance of glycoproteins when administered to an organism, it would be advantageous if one could modify the glycosylation composition and patterns of glycoproteins expressed in heterologous organisms so that they more nearly resemble the naturally occurring glycoproteins. Most, if not all, currently produced

therapeutic glycoproteins are therefore expressed in mammalian cells and much effort has been directed at improving (i.e. humanizing) the glycosylation pattern of these recombinant proteins. Changes in medium composition as well as the co-expression of genes encoding enzymes involved in human glycosylation have been successfully employed (S. Weikert, et al., *Nature Biotechnology*, 1999, 17, 1116-1121).

While recombinant proteins similar to their human counterparts can be made in mammalian expression systems, it is currently <u>not</u> possible to make proteins with a humanoid glycosyation pattern in lower eukaryotes. Although the core oligosaccharide structure transferred to the protein in the endoplasmatic reticulum is basically identical in mammals and lower eukaryotes, substantial differences have been found in the subsequent processing reactions of the Golgi apparatus. This has prevented the use of lower eukaryotes as hosts for the production of recombinant human glycoproteins despite otherwise notable advantages over mammalian expression systems, such as substantially higher product titers, shorter fermentation times, having an alternative for proteins that are poorly expressed in mammaliens cells, and the absence of retroviral infections in such hosts.

The yeast, *Pichia pastoris,* has played a particularly important role as eukaryotic expression system since it is able to grow to high cell densities and secrete large quantities of recombinant protein. However, as noted above, yeast does not glycosylate proteins like higher mammals.

It is therefore an object of the present invention to provide a system and methods for humanizing glycosylation of recombinant glycoproteins expressed in *Pichia pastoris* and other lower eukaryotic protein expression systems.

Summary of the Invention

A protein production system has been developed using (1) a lower eukaryotic host such as a unicellular or filamentous fungus, (2) a higher eukaryotic host such as an insect or plant cell or (3) any eukaryotic cell that has a sufficiently different glycosylation pattern from higher mammals, to modify

the glycosylation composition and patterns to more nearly resemble the naturally occurring glycoprotein. Genes encoding enzymes involved in the processing of glycoproteins are manipulated in the host system for the purpose of "humanizing" the glycosylation pattern of recombinant proteins expressed in such hosts. Genes may be transformed into the host cells expressing the recombinant glycoproteins which encode any combination of the following: sialyltransferases, mannosidases, fucosyltransferases, galactosyltransferases, glucosyltransferases, GlcNAc transferases, ER and Golgi specific transporters (e.g. sym and antiport transporters for UDP-galactose and other precursors), other enzymes involved in the processing of oligosacharides, and enzymes involved in the synthesis of activated oligosachide precursors such as UDP-galactose, CMP-N-acetylneuraminic acid. Genes may be deleted which encode enzymes such as the enzymes homologous to OCH1, MNN1 (putative mannosyltransferases in S. cerevisiae) and other enzymes known to be characteristic of non-human glycosylation reactions.

A process for controlling the glycosylation of protein in a cell wherein the cell is genetically engineered to produce one or more enzymes which provide internal control of the cell's glycosylation mechanism. A Chinese hamster ovary (CHO) cell line is genetically engineered to produce a sialyltransferase. This supplemental sialyltransferase modifies the CHO glycosylation machinery to produce glycoproteins havng carbohydrate structures, which more closely resemble naturally occurring human glycoproteins.

Methods and Reagents Used in the Method

Methods and reagents which can be used in the methods for modifying the glycosylation are described in the literature, such as U.S. Patent No. 5,955,422 "Production of erthropoietin", U.S. Patent No. 4,775,622 "Expression, processing and secretion of heterologous protein by yeast", U.S. Patent No. 6,017,743 Sia.alpha. 2, 3, Gal 1, 4Glc Nac.alpha. 2, 8-sialyltransferase, U.S. Patent No. 4,925,796 "Method for enhancing glycoprotein stability", U.S. Patent No. 5,766,910 "Expression of the developmental I antigen

by a cloned human cDNA encoding a member of the beta-1, 6-N-acetylglucosaminyltransferase gene family", U.S. Patent No. 5,834,251 "Methods of modifying carbohydrate moieties", U.S. Patent No. 5,910,570 "Cloned DNA encoding a UDP-GalNAc: polypeptide N-acetylgalactosaminyltransferase", U.S. Patent No. 5,849,904 "Isolated nucleic acid molecules which hybridize to polysialyl transferases", U.S. Patent No. 5,955,347 "Methods and products for the synthesis of oligosaccharide structures on glycoproteins, glycolipids, or as free molecules, and for the isolation of cloned genetic sequences that determine these structures", U.S. Patent No. 5,962,294 "Compositions and methods for the identification and synthesis of sialyltransferases", U.S. Patent No. 5,135,854 "Methods of regulating protein glycosylation", U.S. Patent No. 4,935,349 "Expression of higher eucaryotic genes in Aspergillus", U.S. Patent No. 5,707,828 "Expression of human serum albumin in Pichia pastoris", and U.S. Patent No. 5,047,335 "Process for controlling intracellular glycosylation of proteins".

Appropriate yeast expression systems can be obtained from sources such as the American Type Culture Collection, Rockville, MD. Vectors are commercially available from a variety of sources.

We claim:

1. A process for altering the glycosylation of a heterologous protein produced by a non-mammalian eukaryotic cell, the process comprising the steps of:

introducing into the non-mammalian eukaryotic cell at least one gene encoding at least one enzyme expressed in mammalian cells which is selected from the group consisting of glycosyltransferase, fucosyltransferases, galactosyltransferases, -acetylgalactosaminyltransferases, N-acetylglycosaminyltransferases and sulfotransferases;

wherein a sufficient amount of at least one of the enzymes is expressed in the eukaryotic cell to alter the glycosylation of the heterologous protein in the eukaryotic cell to resemble the glycosylation composition or pattern of the native protein expressed in mammalian cells.

2. The process of claim 1 wherein the non-mammalian cells are yeast cells.

3. The process of claim 1 wherein the mammal is a human.